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Subdivision of the lateral plate mesoderm and specification of the forelimb and hindlimb forming domains

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ABSTRACT

The limbs are a significant evolutionary innovation that enabled vertebrates to diversify and colonise new environments. Tetrapods have two pairs of limbs, forelimbs in the upper body and hindlimbs in the lower body. The morphologies of the forelimbs and hindlimbs are distinct, reflecting their specific locomotory functions although they share many common signalling networks that regulate their development. The paired appendages in vertebrates form at fixed positions along the rostral–caudal axis and this occurs as a consequence of earlier subdivision of the lateral plate mesoderm (LPM) into regions with distinct limb forming potential. In this review, we discuss the molecular mechanisms that confer a broad region of the flank with limb-forming potential and its subsequent refinement into distinct forelimb-forming, hindlimb-forming and interlimb territories.

Contents

1. Introduction

Tetrapods form two pairs of appendages, the forelimbs and the hindlimbs, at fixed positions along the rostro–caudal body axis. The axial skeleton in vertebrates consists of several types of vertebrae, cervical (neck), thoracic (chest), lumbar (lower back) and sacral (hip). Forelimbs are formed at the cervical–thoracic boundary and hindlimbs at the lumbar–sacral boundary. This relative position of the limbs and vertebrae is conserved despite of the difference in the number of vertebrae in each region in different species [1]. For example, the chicken has 13 cervical and 7 thoracic vertebrae and the mouse has 7cervical and 13 thoracic vertebrae, however the forelimbs are formed at the cervical–thoracic boundary in both species.

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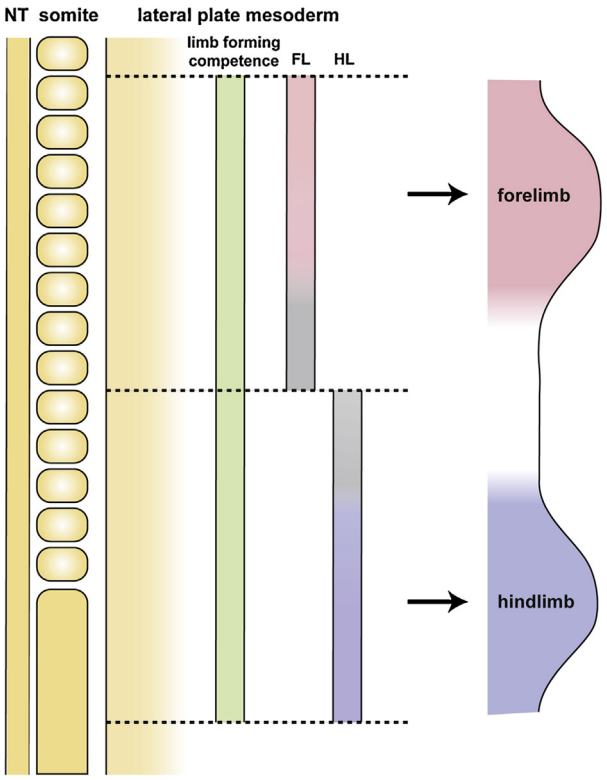

Fig. 1. LPM is divided into subdomains. A broad region of the LPM has a limb forming competence (green), a rostral domain of which has a forelimb forming competence (pink) and a caudal domain of which has a hindlimb forming competence (purple).

The limb developmental programme starts in discrete regions of the LPM (Fig. 1), following an inductive signal from the paraxial mesoderm [2]. Responding to this axial signal, cells in distinct subdomains of the LPM activate genes required to initiate limb outgrowth. These include the T-box transcription factors, *Tbx5* in the forelimb and *Tbx4* in the hindlimb region (discussed in detail in the following sections). *Tbx5* and *Tbx4* establish *Fgf10* expression in the mesenchyme, which subsequently signals to the overlaying ectoderm to activate *Fgf8* transcription. *Fgf8* in turn signals to the mesenchyme to positively regulate *Fgf10* thereby establishing a positive feedback loop [3–7]. This feedback loop of FGF signalling is required and sufficient for both forelimb and hindlimb outgrowth. *Fgf10* mutant mice lack all the limb skeletal elements of autopod, zeugopod and stylopod [4–6] and the phenotype is equally penetrant in forelimb and hindlimb, indicating that while the upstream mechanisms to ensure the establishment of *Fgf10* expression in the forelimb and hindlimb may differ, the objective of establishing *Fgf10* expression and its action are the same. In spite of the common signals shared in forelimb and hindlimb development, limb elements with distinct morphologies are produced. The differences in how forelimb and hindlimb-forming cells will respond to common patterning signals is established early, prior to overt limb bud formation and is a property that is retained even if forelimb cells are grafted into the hindlimb, or vice versa [8]. Here we review the studies that revealed how LPM is divided into subdomains, such as the forelimb forming and the hindlimb forming regions.

2. *Tbx5* and *Tbx4* serve as markers of the subdomains within the LPM

The clearest gene molecular marker of whether cells will produce forelimb, or hindlimb structures are the T-box transcription factors, *Tbx5* and *Tbx4* and a paired-type homeodomain transcription factor, *Pitx1* and a LIM-homeodomain transcription factor, *Islet1* [9–14]. *Tbx5* expression is restricted to the forelimb forming LPM whereas *Tbx4*, *Pitx1* and *Islet1* are restricted to the hindlimb forming LPM.

Tbx5 and *Tbx4* are paralogous genes derived from an ancestral *Tbx5/4* gene. These genes play essential roles in the initiation of limb outgrowth. Both *Tbx5* mutants and *Tbx5* morphants of zebrafish fail to form pectoral fins, the homologous structure of the forelimb [15–17] Furthermore, deletion of *Tbx5* in mouse results in the loss of all the forelimb skeletal elements [18,19]. In human, mutations in *TBX5* are associated with Holt–Oram Syndrome (HOS; OMIM 142900), a dominant disorder characterized by heart and upper limb abnormalities [20,21]. The skeletal abnormalities in the upper limb range from mild triphalangeal thumb to phocomelia in severe cases. These studies demonstrated a conserved role of *Tbx5* in forelimb formation.

Similarly genetic deletion of mouse *Tbx4* leads to outgrowth defects of the hindlimb, although some rudimentary distal structures are formed [22]. This suggests that *Tbx4* is essential for normal hindlimb initiation, however its requirement is not exclusive as *Tbx5* in the forelimb and other factors function redundantly. Mutations in human *TBx4* are associated with Small Patella syndrome (SPS; OMIM 147891), a dominant disorder characterized by dysplasia of patella, pelvis and foot [23].

The restricted expression domains of *Tbx5* and *Tbx4* in the forelimb and the hindlimb, respectively, suggest that these genes could play an active role in determining forelimb and hindlimb morphologies and this was supported by some experiments in the chick [24,25]. Gene deletion–gene replacement experiments in mouse embryos, however, clearly demonstrated that *Tbx5* and *Tbx4* have equivalent roles in the initiation of limb outgrowth and do not control limb-type specific morphology [26]. Ectopic expression of *Tbx4* in the *Tbx5* mutant forelimb can rescue forelimb formation in the absence of *Tbx5* activity demonstrating that *Tbx4* can produce forelimb features and *Tbx5* is not required for forelimb structures to form. There is good evidence, however, that the hindlimb-restricted gene, *Pitx1*, can determine at least some aspects of hindlimb-specific morphology. Forelimbs expressing *Pitx1* ectopically acquire hindlimb-like morphology in chick and mouse embryos [25,27,28]. A similar activity is apparently observed in humans. Liebenberg syndrome (OMIM 186550) is thought to be caused by regulatory mutations in *Pitx1*, causing it to be expressed ectopically in the forelimb. Individuals with Liebenberg syndrome have long arms, elongated metacarpals and dramatically affected elbow joints that have features similar to a knee joint, including a patella [29]. In the mouse, the relatively longer hindlimb metatarsals compared to forelimb metacarpals are generated by increasing the growth rates of the metatarsal primordia during a discrete time-window [30]. This accelerated growth of the metatarsals is regulated by *Pitx1* and the growth rate of metacarpal elements can be made metatarsal-like by ectopic expression of *Pitx1* in the forelimb [30].

The correlation between the expression profile of *Tbx5* and *Tbx4* and the type of limb these cells go on to form has been demonstrated using ectopically induced limbs in the chick inter-limb LPM. A bead soaked with FGF can induce a wing-like structure when placed near the endogenous wing and this ectopic limb bud expresses *Tbx5*, while an FGF bead placed near the endogenous leg can induce a leg-like structure that expresses *Tbx4* [12,13,31]. Perhaps clearest of all are ectopic limbs induced from the middle of the interlimb that have mosaic morphology, the anterior part closest to the wing forms wing digits while the posterior part closest to the hindlimb forms leg digits and this is reflected in the domains of *Tbx5* and *Tbx4*/*Pitx1* expression, which are restricted to the anterior and posterior parts of the ectopic limb buds. These results demonstrate that *Tbx5* and *Tbx4* are markers of the forelimb and hindlimb

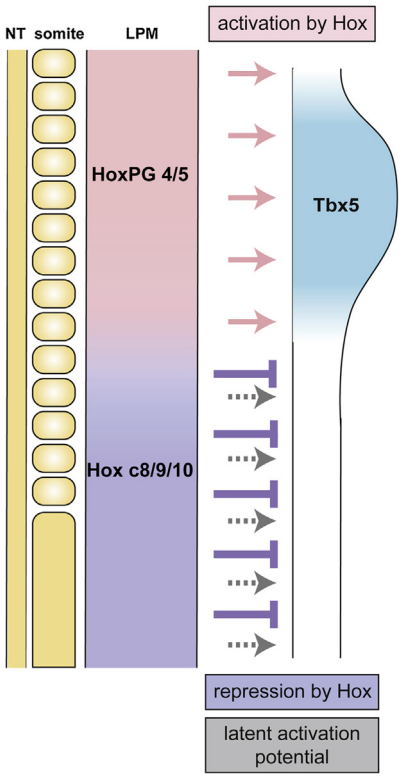

Fig. 2. Regulation of forelimb-restricted *Tbx5* expression by Hox gene. Hox genes expressed in the forelimb-forming LPM, such as Hox PG 4 and 5, induce *Tbx5* expression (pink arrows). In the caudal LPM there is a latent potential to express *Tbx5* (grey arrows) that is normally masked by the presence of *Hox c8–10* genes (purple arrows). Thus, a combination of Hox collinear expression along the A–P axis and the specific activator or repressor activities of distinct Hox protein paralogues dictates positioning of the forelimb-forming region.

progenitors, respectively and can be used to identify the upstream factors that subdivide the LPM to forelimb and hindlimb forming domains.

3. A combination of transcriptional activation and repression by a collinear Hox code restricts *Tbx5* expression to the forelimb.

The analysis of the *cis*-regulatory element of *Tbx5* has identified a number of upstream regulators, including Hox genes, β-catenin and retinoic acid (RA) signalling and this has revealed the molecular network that determines the forelimb LPM [2,32,33].

Hox genes are an evolutionarily conserved homeodomain containing transcription factors found in clusters in the genome. In mammals, there are four clusters, *HoxA*, *HoxB*, *HoxC* and *HoxD*, resulting in two to four members for each paralogous groups (PGs) that share some redundant functions [34]. Their organization in the chromosome correlates with their expression domains along the rostro–caudal axis of embryos and the time of their expression; genes located in the 3′ of the cluster are expressed in the rostral regions at early stages of development and the ones sequentially more 5′ are expressed later in successively more caudal regions.

Analysis of the *Tbx5 cis*-regulatory element in intron2 has identified the direct input of Hox genes in determining the position of the *Tbx5* expression domain along the rostral–caudal axis [32,33] (Fig. 2). Hox PG4 and 5 genes are expressed in the forelimb-forming LPM and positively regulate *Tbx5* transcription. In contrast, Hox genes located more 5′ in the cluster, such as *Hoxc8*, *c9* and *c10* are expressed exclusively in the caudal LPM where *Tbx5* is not normally expressed and these genes actively repress *Tbx5* transcription. This establishes a boundary between *Tbx5*-positive rostral LPM that forms the forelimb and *Tbx5*-negative caudal LPM including the hindlimb forming region. Both activation and repression of *Tbx5* are regulated by direct binding of Hox proteins to the *Tbx5* regulatory element. A short *cis*-regulatory fragment that is capable of recapitulating the forelimb-restricted expression domain of *Tbx5*, contains 6 Hox binding sites. 5 sites are required for activation of the regulatory element while a single site is required for repression of *Tbx5* from domains of the LPM caudal to the forelimb-forming region.

The hindlimb *cis*-regulatory elements of *Tbx4* have been mapped to 2 regions one 5′ and the other in the 3′ of the coding exons [35]. Detailed analysis of the key transcription factor binding sites have not been reported to date. Although future studies are required, it is tempting to speculate that a combination of positive and negative control by Hox genes restricts *Tbx4* expression to the hindlimb LPM in a similar manner to *Tbx5* in the forelimb LPM.

4. Spatial regulation of *Pitx1* expression

Pitx1 expression is restricted to the hindlimb and *Hoxc9* can regulate its transcription [33] (Fig. 3C). Mis-expression of *Hoxc9* in the forelimb LPM induces ectopic *Pitx1* expression. As *Hoxc9* expression is restricted to the caudal LPM similar to *Pitx1*, a positive regulation by *Hoxc9* may be a mechanism of hindlimb LPM-specific *Pitx1* expression. These results indicate that *Hoxc9* can act both as a transcriptional activator (to regulate *Pitx1*) and a repressor (to regulate *Tbx5*). The transcriptional activity of Hox genes is, at least in part, regulated by Hox cofactors. TALE class homeodomain proteins, Pbx and Meis, can modify the activity of Hox complexes by controlling accessibility of histone modification enzymes [36,37] and another homeodomain protein, Engrailed (En), can mediate the transcriptional repression [38]. Identification of *Pitx1* regulatory element will reveal the mechanisms controlling the transcriptional activity of *Hoxc9* in the LPM.

The expression profile of *Pitx1* is different from those of *Tbx5* and *Tbx4* at early stages. In chick embryos, *Pitx1* expression starts broadly in the LPM at pre-limb bud stages, strongly expressed in the caudal LPM and weakly in the rostral LPM including the forelimb forming regions (Fig. 3A). Subsequently, the expression is restricted to the hindlimb LPM (Fig. 3B). This expression pattern is distinct from those of *Tbx5* and *Tbx4* that are restricted to the forelimb and the hindlimb LPM, respectively. Down-regulation of *Pitx1* in the rostral LPM suggests either a transcriptional repressive mechanism operating in the region, or a restriction of an upstream regulator. Since its expression in the caudal LPM is maintained, any repressive machinery must be functionally specific to the rostral LPM. Obvious candidates are Hox genes, such as Hox PG4 and 5. This opens the possibility of PG4/5 genes forming either repressive or activating complexes on the regulatory sequences of different target genes.

5. Establishment of collinear Hox expression is controlled at multiple levels.

Analysis of the controlled *Tbx5* expression in the LPM demonstrates how positional information encoded by nested Hox expression along the body axis is interpreted to subdivide the LPM. Thus, the establishment of the collinear Hox code is essential to specifying the forelimb and the hindlimb forming domains at the correct position. A wide range of mechanisms are involved in assuring the robust expression patterns of Hox genes. Since recent findings on the mechanisms regulating collinear Hox expressions have been reviewed elsewhere [39,40], here we only describe some features of its regulation.

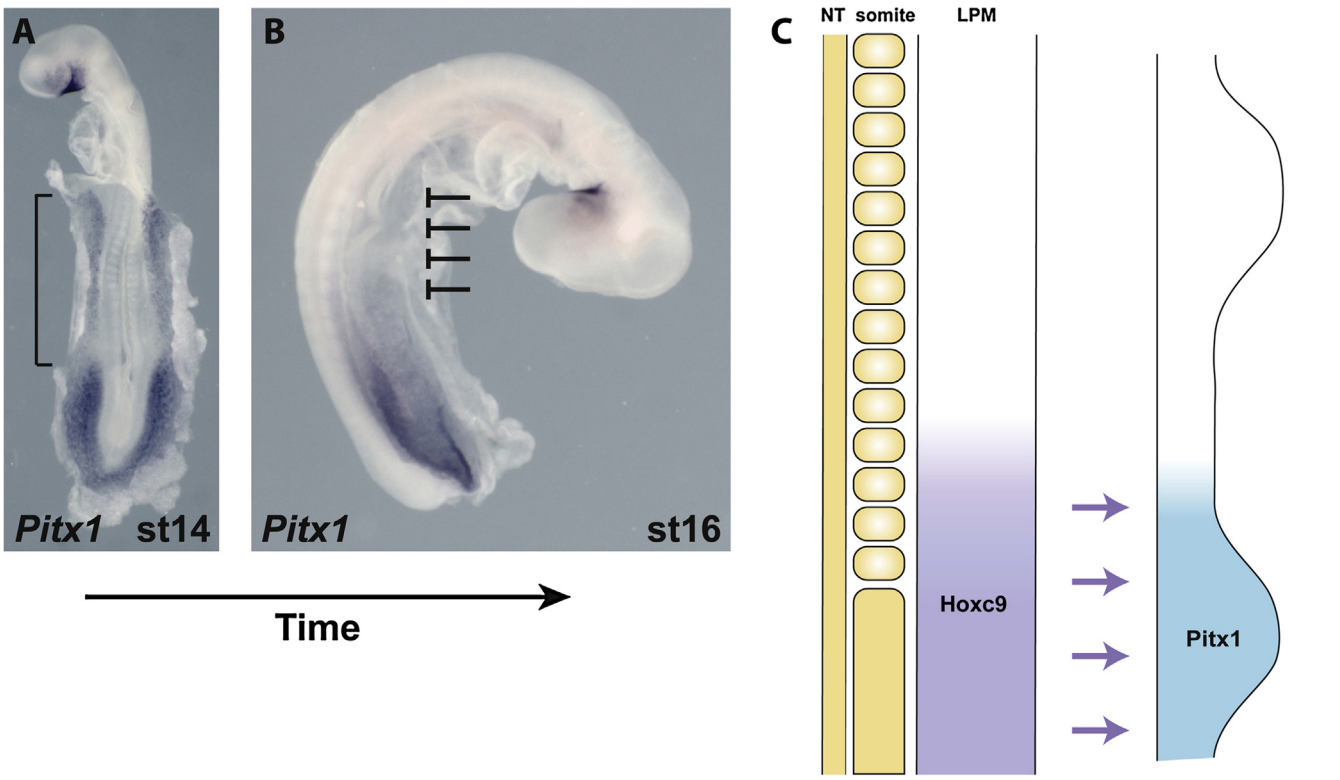

Fig. 3. Regulation of *Pitx1* expression. A–B. *Pitx1* expression in chick embryos. *Pitx1* is expressed throughout the LPM at stage 14, strongly in the caudal hindlimb forming region and weakly in the rostral region (blacket) (A). Subsequently, the expression is restricted to the hindlimb forming region at stage 16 (B). (C). *Pitx1* transcription is positively regulated by Hox genes in the caudal LPM, such as *Hoxc9*. Since *Hoxc9* expression is restricted to the caudal domain including the hindlimb forming regions, this may be one of the regulatory mechanisms of restricted *Pitx1* activation.

The regulation of Hox gene transcription during axis development is divided into at least two phases. At the initial stages of development, Hox genes are kept silent and activated sequentially starting from the genes located in the 3′ end of the cluster continuing to the ones located at the more 5′ end (temporal collinearity). This process is controlled globally by a balance between a repressive influence from the 5′ side of the cluster and a positive influence from the 3′ side, and the timing of activation is determined in a distance dependent manner [41]. Subsequently, the domains of Hox gene expression are refined to produce the collinear order of expression domains with 3′ Hox genes in the rostral regions and 5′ Hox genes in the caudal regions (spatial collinearity). This nested expression pattern is determined by local regulatory elements [41].

Analyses of chromatin architecture demonstrated a correlation between the distinct subsets of Hox genes expressed at different A–P domains and a dynamic 3D chromatin conformation [42,43]. The Hox genes are divided into two distinct 3D compartments; inactive 5′-located Hox genes marked by H3K27me3 are organized in a local compartment and active genes located at more 3′ position marked by H3K4me3 are in the other. This bimodal organization results in a physical separation between active and inactive Hox genes. The boundary between these two compartments is at different positions between the rostral and caudal trunk, it is located at 3′ of the cluster in the anterior trunk and shifted to 5′ of the cluster in the posterior trunk, indicating a sequential transition of each gene from a negative to a positive compartment.miRNAs also regulate Hox expression (reviewed in [44]). miRNAs are small (around 22 nucleotides) non-coding RNAs that bind to target sequences in the 3′ UTR of mRNAs to promote mRNA degradation or repress translation. In vertebrates, miRNA-196 and miRNA-10 are located within the Hox clusters and down-regulation of these miRNAs causes upregulation of Hox expression *in vivo* [45–47], suggesting the importance of their regulation at post-transcriptional level.

6. Specification of limb forming LPM by the β-catenin pathway

Another regulator of *Tbx5* is β-catenin/TCF/LEF, one of the mediator complexes of the Wnt signalling pathway. The canonical Wnt-β-catenin pathway plays a pivotal role in a number of processes including cell fate specification and cell proliferation by regulating the transcription of target genes.

A requirement for the Wnt-β-catenin pathway in early limb development and for the endogenous expression of *Tbx5* has been shown in zebrafish and chick embryos [16]. The role of this pathway in mouse limb induction and initiation has, however, been less clear because limb buds formed even in the absence of *LEF1* and *TCF1* [48]. In addition, candidate Wnt ligands involved in this process have not been identified in mouse. Activation of Wnt-β-catenin pathway, however, is higher in the limb forming LPM than the interlimb LPM at pre-limb bud stages, suggesting a role for canonical Wnt signalling in limb initiation [49]. The role of β-catenin in limb formation was further demonstrated by deleting β-catenin from the presumptive hindlimb, which inhibited hindlimb outgrowth. In addition, β-catenin is required for the maintenance of *Islet1* expression. A direct requirement of β-catenin signalling for *Tbx5* transcription was clearly revealed by the presence of an essential TCF/LEF binding site in the *Tbx5* forelimb regulatory element [2]. Furthermore, forelimb outgrowth defects in β-catenin mutants can be rescued by misexpression of *Tbx5*. These results seem inconsistent with the *TCF1*/*LEF1* double mouse mutant phenotype, however, weak expression of other genes such as *TCF3* and *TCF4* from the *TCF*/*LEF* family, may be sufficient to initiate the limb programme.

The Wnt ligands involved in mouse limb induction and initiation are still not clear. Although *Wnt2* is expressed in the LPM at pre-limb bud stages [2,50], deletion of *Wnt2* does not cause any obvious limb defects [50], suggesting that other functionally redundant Wnt

ligands may also be required. However, in chicken embryos, there is a clear correlation between differential Wnt ligand expressions and the subdomains of the LPM, *Wnt2b* in the forelimb forming LPM and *Wnt8c* in the hindlimb forming LPM [51]. But the expression of *Wnt2b* and *Wnt8c* are not conserved in the mouse.

7. Specification of the limb forming LPM by retinoic acid signalling

Retinoic acid (RA) is essential in embryonic development. RA controls a wide range of target genes by binding to nuclear receptors, RAR and RXR [52]. The requirement for RA in limb development has been demonstrated in zebrafish, chick and mouse embryos [53–58]. However, there is a controversy regarding whether RA signalling is required cell-autonomously or cell-non-autonomously in forelimb formation. Mosaic analysis in zebrafish suggests that RA signalling is required cell autonomously [59]. This model is supported by a promoter analysis of mouse *Tbx5*, demonstrating that RA signalling directly regulates *Tbx5* transcription [2]. In contrast, the studies using *RARE-reporter* in the mouse suggests that RA signalling is required in the body axis, but not in the limb forming LPM, and regulates *Tbx5* indirectly through repression of *Fgf8* [60,61]. The same study also showed that RA signalling is not required for hindlimb bud formation [61]. In contrast, chick experiments using a chemical inhibitor of RA signalling demonstrated the requirement of RA in *Tbx4* transcription [2]. This discrepancy may be because of the sensitivity of the reporter strain they used so that it does not detect a low level of maternal RA although we cannot exclude the possibility that the requirement of RA is slightly different in mouse and chick embryos.

In wild type embryos the LPM exposed to RA signalling is not restricted to the limb forming regions [61]. Therefore, it is likely that RA functions as a permissive factor that confers cells in a broad region of the LPM with limb forming potential.

8. Rostro–caudal patterning of axial tissues and LPM is coordinated to ensure the relative position of axial structures and limbs

The limbs are formed at a fixed position along the rostro–caudal body axis and the relative position of the limbs and vertebrae is conserved despite of the difference in the number of vertebrae in each region in different species [1]. This raises the question how regionalization of the LPM is coordinated with that of axial tissues and whether "positional information" in the LPM is under the influence of axial A–P patterning.

Vertebrae are derived from the somites and the morphologies specific to each vertebra are determined by Hox genes. Genetic manipulations in mouse embryos demonstrated that both deletion and ectopic expression of Hox genes cause homeotic transformation [62]. The defects tend to be more severe if more than one gene from the PG are mutated simultaneously because of their functional redundancy. In addition to axial transformation, some mutants also displayed dislocated limbs [63,64], like the disruption of *Hoxb5* cause a rostral shift of the forelimb [63] and disruption of genes from *Hox8 PG* produces caudal shifted hindlimbs [64]. Since the hindlimbs are attached to the axial skeleton at sacral regions and *Hox8 PG* mutants display transformation of the 1st sacral vertebra to lumbar vertebra-like morphology, the caudal shift of the hindlimb is associated with the position of the sacrum. These results suggest a role for Hox genes in limb positioning. It is not clear from these studies, however, whether the Hox code in axial tissues has some influence on limb positioning since these mutants are conventional mutants and the expressions of Hox genes are altered throughout the embryo including the LPM.

When *Hoxb6* is misexpressed in the presomitic mesoderm and newly formed somites, ectopic ribs form throughout the whole length of the axial skeleton rather than being restricted to the thoracic region, suggesting certain Hox PG genes have a role in controlling thoracic specific morphology [65]. Strikingly, they also display a rostral shift of the forelimbs. This result demonstrates that the change of Hox code in the paraxial mesoderm is sufficient to alter limb position, however, how the positional information encoded by the Hox code in the axial tissues influences Hox gene expression in the LPM remains unclear. These results suggest that a cue from the axial tissues to the LPM is involved in adjusting the limb position to ensure the relative position of the axial tissues and the limbs. There may be reciprocal cross-talk between axial and LPM that stabilize limb position relative to axial structures.

Gdf11 signalling coordinates processes involved in the trunk-to-tail transition and positioning the hindlimb-forming region [66]. *Gdf11* is a secreted, TGF-β family member and signals through Smad2/3. Deletion of *Gdf11* causes axial transformation. Hox expression domains are shifted caudally with a corresponding caudal shift of hindlimbs in these mutant mice [67]. The activation of this pathway by a constitutively active form of receptor, *Alk5*, in the posterior epiblast and the primitive streak results in a short trunk and rostral shift of hindlimbs [66], suggesting that manipulation of this pathway is sufficient to change the hindlimb position. *Islet1* was identified as a direct target of *Gdf11* signalling and it is suggested that *Islet1*, but not Hox, is a major regulator of hindlimb positioning [66].

9. Emergence of limb forming domains during vertebrate evolution

Acquisition of paired appendages was an innovation that enabled the successful expansion of vertebrates, the colonisation of land and the ability to fly. The evolutionary changes that needed to take place in early vertebrates were therefore highly significant. The extant Cephalochordate, amphioxus, provides an accessible model of an ancestral vertebrate body plan. Amphioxus diverged from other chordates before the genome duplications that occurred twice during vertebrate evolution and thus possesses a single, ancestral *Tbx5/4* gene (*amphi Tbx5/4*). The *amphi Tbx5/4* gene has the capacity to rescue forelimb formation in the *Tbx5* mutant mouse [68]. This indicates that the ancestral gene from the limbless organism has an ability to initiate limb formation programme and suggests that activation of *Tbx5* and *Tbx4* transcription in the appropriate domains of the LPM was a key step in the acquisition of limbs rather than any other novel functions. One scenario is that the expressions of essential regulatory factors were acquired in the limb forming regions. Alternatively, a second model is that mutations were introduced on *cis*-regulatory elements of *Tbx5/4* to respond to the regulatory factors that were already expressed in these regions.

In lamprey, a limbless vertebrate Agnathan, the LPM is divided into cardiac mesoderm (CM) and the posterior LPM located caudally to CM (PLPM), similar to Gnathostome. The ancestral gene of *Tbx5* and *Tbx4*, *LjTbx4/5*, is not expressed in the PLPM and neither of *LjFgf7/10/22*, an ortholog of *Fgf10*, nor *LjFgf8/17*, an ortholog of *Fgf8*, are detected in the PLPM, suggesting that the factors initiating vertebrate limb development are not expressed in the lamprey PLPM [69]. In contrast, *LjHox5i* and *LjHox6w* are expressed in the PLPM and the anterior expression boundary of *LjHox5i* locates rostral to that of *LjHox6w* [69], reflecting spatial collinearity. This demonstrates that the PLPM of the lamprey is patterned by nested expressions of Hox genes and that at least one of the essential factors for *Tbx5* transcription is expressed.

10. Specification of the forelimb forming domain in flightless bird

The avian wing is an example of a forelimb that has been adapted for flight. The size, shape and structure of the wing can all affect flight performance. Flightless birds that have lost this flight ability develop smaller wings than volant birds. Analysis of the temporal and spatial expression domains of *Tbx5* in emu has provided clues to the mechanism that could have modified upper limbs in flightless birds [70]. Interestingly, the size of the *Tbx5* expression domains at pre-limb bud stages is comparable between chick and emu. Instead, *Tbx5* transcription is initiated relatively late in the emu LPM. These patterns suggest that the reduction in the forelimb elements is achieved not by reducing the spatial domain of *Tbx5* but rather by modifying when it is first expressed. A mouse study demonstrates that limb precursor cells respond to *Tbx5* input to initiate limb outgrowth only for a short time period [71]. Therefore, in the emu by delaying when *Tbx5* is activated to initiate forelimb formation, the window during which it can recruit limb precursors is shortened and as a result a smaller cohort of cells form a limb bud that is reduced in size and a smaller (although scaled) limb is produced. Since the spatial regulation of *Tbx5* expression is controlled by Hox genes, modification of timing without affecting the spatial domain may have enabled the emu to adapt forelimb development without affecting the Hox code in the LPM, that could result in a dramatic change throughout the A–P axis of the developing body.

11. Specification of the limb forming domains and heterochrony in marsupials

The relative timing of forelimb versus hindlimb development varies widely between vertebrate clades [72] and one example from mammals is the accelerated forelimb development compared to the hindlimb in marsupials. Marsupials have a short gestation period and neonates are underdeveloped compared to the eutherians. Opossum neonates are born at the developmental stage equivalent to 10–12 weeks of human embryos. Strikingly, however, the forelimbs are functional with fully developed muscular and nervous systems, which are used to crawl from the birth canal to the teat. The onset of opossum *Tbx5* expression is earlier than the mouse and its expression domain is bigger [73,74], suggesting that the accelerated forelimb development is achieved by heterochoronical and heterotopical regulation of the forelimb forming domain. The expanded domain of *Tbx5* expression can be explained by shifted rostro–caudal Hox code [73], while the mechanism of its early expression is unclear. Interestingly *Tbx4* in the hindlimb also comes on earlier than the mouse, however, the subsequent developmental programmes proceed slower; *Fgf8* is expressed at similar stages to the mouse and the hindlimbs are small and underdeveloped in the neonates in contrast to the forelimb [73]. Comparison of mammalian forelimb morphologies suggests that the heterochrony exhibited by the opossum limb does not drive any obvious morphological disparity [75].

12. Limb loss in vertebrates

In the python there is evidence that the mechanism of forelimb loss is caused as a result of altering the nested expression of Hox genes in the LPM [76]. This can be viewed effectively as a respecification or failure to specify the future forelimb forming subdomains of the LPM, causing the loss of forelimb. In contrast, the hindlimb buds are initiated, but its outgrowth is not maintained and rudimentary hindlimbs are formed, suggesting that regression of the forelimbs and the hindlimbs occurred independently. This is consistent with the evidence in the fossil record that forelimbs were lost before the hindlimbs in *Pachyrhachis*, an extinct snake that has no forelimbs but retains hindlimbs. An extant example of uncoupled forelimb and hindlimb limb loss is provided by the amphisbaenian reptile *Bipes Biporus* (the mole lizard). This species has forelimbs but no external hindlimbs, and only a remnant of the pelvic girdle remains. An example of limb loss in mammals is the hindlimb loss in cetaceans (Dolphins). Cetacean embryos form the hindlimb buds, however *Shh* is not expressed in the ZPA and the AER is not maintained. Since python hindlimbs lack *Shh* expression in the ZPA and do not form an AER, it has been suggested that these two species may use a similar mechanism to reduce the hindlimbs [76,77].

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